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Fas Modulation of Apoptosis during Negative Selection of Thymocytes

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Summary

A major mechanism maintaining immune tolerance is the deletion of potentially autoreactive thymocytes by apoptosis during development in the thymus. Previous reports suggest that apoptosis is induced by high avidity signals transduced via the T cell receptor; however, the role of signals transduced by other cell surface receptors during thymic selection remains poorly understood. Fas, a member of the TNF receptor family, has been shown to induce apoptosis in mature peripheral T cells; however, the effects of Fas on negative selection of thymocytes have not been previously detected. Using a sensitive terminal deoxynucleotidyl transferase method to detect apoptotic cells, we found that mutant Fas molecules in *lpr* mice decrease the sensitivity of thymocytes to T cell receptor–mediated apoptosis and that blockade of Fas–Fas ligand interactions in vivo can inhibit antigen-induced apoptosis of thymocytes in non-*lpr* mice. Thus, we have shown that Fas, in conjunction with antigen-specific signals, can modulate apoptosis during negative selection of thymocytes.

Introduction

Current models of thymic selection propose that the fate of thymocytes (positive or negative selection or neglect) is primarily determined by the avidity of the T cell receptor (TCR) interaction with major histocompatibility complex (MHC) plus peptide antigen (Jameson et al., 1995). This is supported by observations that altered peptide ligands, or differing concentrations of a single peptide, can switch the fate of thymocytes from negative to positive selection (Ashton-Rickardt et al., 1994; Hogquist et al., 1994; Jameson et al., 1994). However, cell surface receptors in addition to the TCR are likely to have important roles in determining the fate of thymocytes (Page et al., 1993). For example, the CD4 and CD8 coreceptors have been shown to effect thymic development owing to both signal transduction and changes in cell–cell avidity (Killeen and Littman, 1996). Adhesion due to LFA-1–ICAM-1 interactions has been shown to be necessary for the generation of double positive (DP) thymocytes (Fine and Kruisbeek, 1991). Apoptosis induced by TCR signals has been reported to require a costimulatory signal provided by CD28 (Punt et al., 1994). Two receptor–ligand pairs of the tumor necrosis factor receptor (TNFR)/TNF families, CD30–CD30 ligand and CD40–gp39, have been shown to modulate negative selection of thymocytes (Amakawa et al., 1996; Foy et al., 1995).

Fas (CD95), another member of the TNFR family, is expressed on most thymocytes. Previous reports have shown that apoptosis of peripheral T cells by activation-induced cell death requires Fas signals in addition to TCR triggering (Brunner et al., 1995; Dhein et al., 1995; Ju et al., 1995); however, the role of Fas during thymic selection has not been established. In addition, the expression of Fas ligand (FasL) in the thymus is controversial (Nagata and Suda, 1995; Suda and Nagata, 1994; Suda et al., 1993). It has been demonstrated that anti-Fas monoclonal antibody (MAb) induces apoptosis of thymocytes in vitro (Ogasawara et al., 1995); however, it has been proposed by several groups that Fas is not actually functional during thymic selection (Adachi et al., 1996; Giese and Davidson, 1992; Herron et al., 1993; Kotzin et al., 1988; Mountz et al., 1990; Musette et al., 1994; Sidman et al., 1992; Singer and Abbas, 1994; Singer et al., 1994; Singer et al., 1989; Sytwu et al., 1996). This interpretation is based upon analyses of the TCR repertoire of *lpr* mice, a mutant strain lacking detectable expression of Fas protein. In these reports, *lpr* mice undergo the predicted deletion of TCR Vβ chains in response to endogenous superantigens. In contrast, our current results indicate that interactions between Fas and FasL can modulate the induction of apoptosis of thymocytes during negative selection. Specifically, we show that thymocytes from *lpr* mice have a decreased susceptibility to TCR-induced apoptosis and that antigen-specific deletion of thymocytes in normal mice can be inhibited in vivo by blocking the Fas–FasL interaction. Our results show that Fas–FasL interactions can also modulate apoptosis of thymocytes.

Results

A hallmark of apoptosis of many cell types, including thymocytes, is the activation of endogenous endonucleases that cleave the genomic DNA into oligonucleosomal-sized fragments (Wyllie, 1980). To detect apoptotic cells, we have adapted methods that use the enzyme terminal deoxynucleotidyl transferase (TdT) to incorporate fluorescent nucleotides onto the DNA fragments

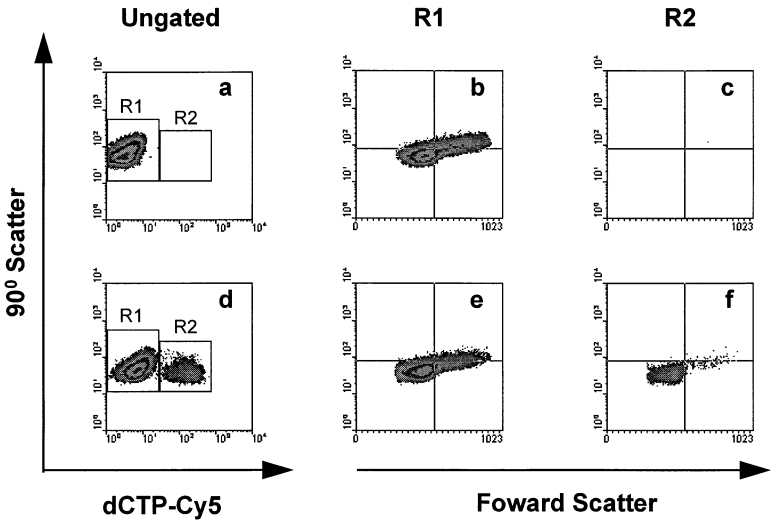

Figure 1. Detection of Apoptotic Thymocytes within a Normal Lymphocyte Scatter Profile

BALB/c thymocytes were incubated in media at 4°C (a–c) or 10^{-6} M dexamethasone (d–f) for 1.5 hr, and cells were then stained with TdT plus dCTP–Cy5 and analyzed by flow cytometry. Results show dCTP–Cy5 (X axis) and 90° scatter (Y axis) of viable (gate R1) and apoptotic (gate R2) cells (a and d), forward scatter (X axis) and 90° scatter (Y axis) of viable (gate R1; b and e) and apoptotic (gate R2; c and f) cells. We collected 2×10^4 events with a Becton Dickinson Vantage using Lysis software. List-mode data were analyzed with Winmidi software. Data are representative of three independent experiments.

in apoptotic cells, rendering them detectable by flow cytometry (Gavrieli et al., 1992; Gorczyca et al., 1992). dCTP incorporation in freshly isolated control BALB/c thymocytes or thymocytes cultured in media at 4°C for 1.5 hr was <1% (Figure 1a). However, after incubation of BALB/c thymocytes for 1.5 hr in dexamethasone, 34% of the cells are apoptotic based on increased incorporation of dCTP (Figure 1d). During apoptosis, phenotypic changes include decreases in cell size (detected by decreased forward scatter) and increases in membrane blebbing (detected by increased 90° scatter). Analysis of the forward and 90° scatter profiles of the viable (gate R1) and apoptotic (gate R2) thymocytes from the control group showed a normal scatter profile of the viable cells (Figure 1b) and an insufficient number of apoptotic cells to analyze (Figure 1c), whereas the dexamethasone-treated group showed that the apoptotic cells (Figure 1f) had a decreased size (mean forward scatter = 459) compared with the viable population (Figure 1e) (mean forward scatter = 483). Importantly, the size and 90° scatter of both the normal and apoptotic populations were within the normal scatter profiles of unmanipulated thymocytes, indicating that DNA strand breaks can be detected by TdT labeling before cells undergoing apoptosis become smaller than viable cells. At later timepoints, for example 4–8 hr, a population of small apoptotic cells with forward scatter values less than the viable population was detected (data not shown). Thus, these results indicate that TdT labeling is a sensitive method capable of detecting cells while they are still within the normal thymocyte scatter profile.

Thymocytes from Fas-Defective *lpr* Mice Have Decreased Sensitivity to Apoptosis

The role of Fas in regulating apoptosis of thymocytes was analyzed by comparing the induction of apoptosis in MRL-$^{+/+}$ (MRL-$^{+}$) and MRL-*lpr*/*lpr* (MRL-*lpr*) thymocytes (Figure 2). MRL-*lpr* mice do not express detectable levels of Fas owing to a retrotransposon insertion that disrupts correct splicing of Fas mRNA (Adachi et al., 1993). Our results indicate significant differences between the induction of apoptosis in *lpr* and normal mice using the sensitive TdT labeling method to detect apoptotic cells. After incubation in media at 37°C, the level of spontaneous apoptosis was 13.5% in MRL-$^{+}$ (Figure 2b) versus only 4.3% in MRL-*lpr* thymocytes (Figure 2i), whereas at 4°C, a temperature that inhibits the apoptotic process, we detected <1.5% apoptotic cells in both strains, which is similar to freshly isolated thymocytes (Figures 2a and 2h). The level of spontaneous apoptosis was not affected in either the MRL-*lpr* or MRL-$^{+}$ strains by the addition of the control human immunoglobulin G1 (IgG1) L6 MAb (Figures 2c and 2j) or Fas–Fc, a fusion protein composed of the extracellular domain of Fas linked to the Fc region of human IgG1 (Figures 2d and 2k). The fact that Fas–Fc did not inhibit spontaneous apoptosis in either strain suggests that spontaneous apoptosis in vitro was due, at least in part, to Fas-independent signals. In addition, the increase in spontaneous apoptosis by the MRL-$^{+}$ compared with the MRL-*lpr* thymocytes may be partially due to apoptotic signals received in vivo prior to harvesting, and thus resistant to in vitro inhibition by Fas–Fc. After incubation with immobilized anti-CD3ϵ MAb 2C11, the level of apoptosis increased to 24.6% in the MRL-$^{+}$ (Figure 2e) compared with only 12.2% in the MRL-*lpr* thymocytes (Figure 2l). Importantly, blocking Fas–FasL interaction with the Fas–Fc fusion protein inhibited the 2C11-induced apoptosis in the MRL-$^{+}$ thymocytes (Figure 2g), whereas control L6 MAb had no effect (Figure 2f). Neither Fas–Fc or L6 had inhibitory effects on the smaller increase in 2C11-induced apoptosis in MRL-*lpr* thymocytes (Figures 2n and 2m). In contrast, dexamethasone produced comparable increases above spontaneous levels of apoptosis of 18.8% in MRL-$^{+}$ and 22.1% in MRL-*lpr* thymocytes, and, consistent with previous reports, Fas–Fc did not inhibit dexamethasone-induced apoptosis in either strain (data not shown). These results suggest that Fas–FasL interactions modulate apoptosis after TCR cross-linking.

MRL-$^{+}$ thymocytes shown in Figures 2a–2g were examined for the expression of Fas (Figures 2o–2u). Previous results have shown that Fas is expressed at high levels on late DP, at lower levels on more mature single positive (SP), and at undetectable levels on most double

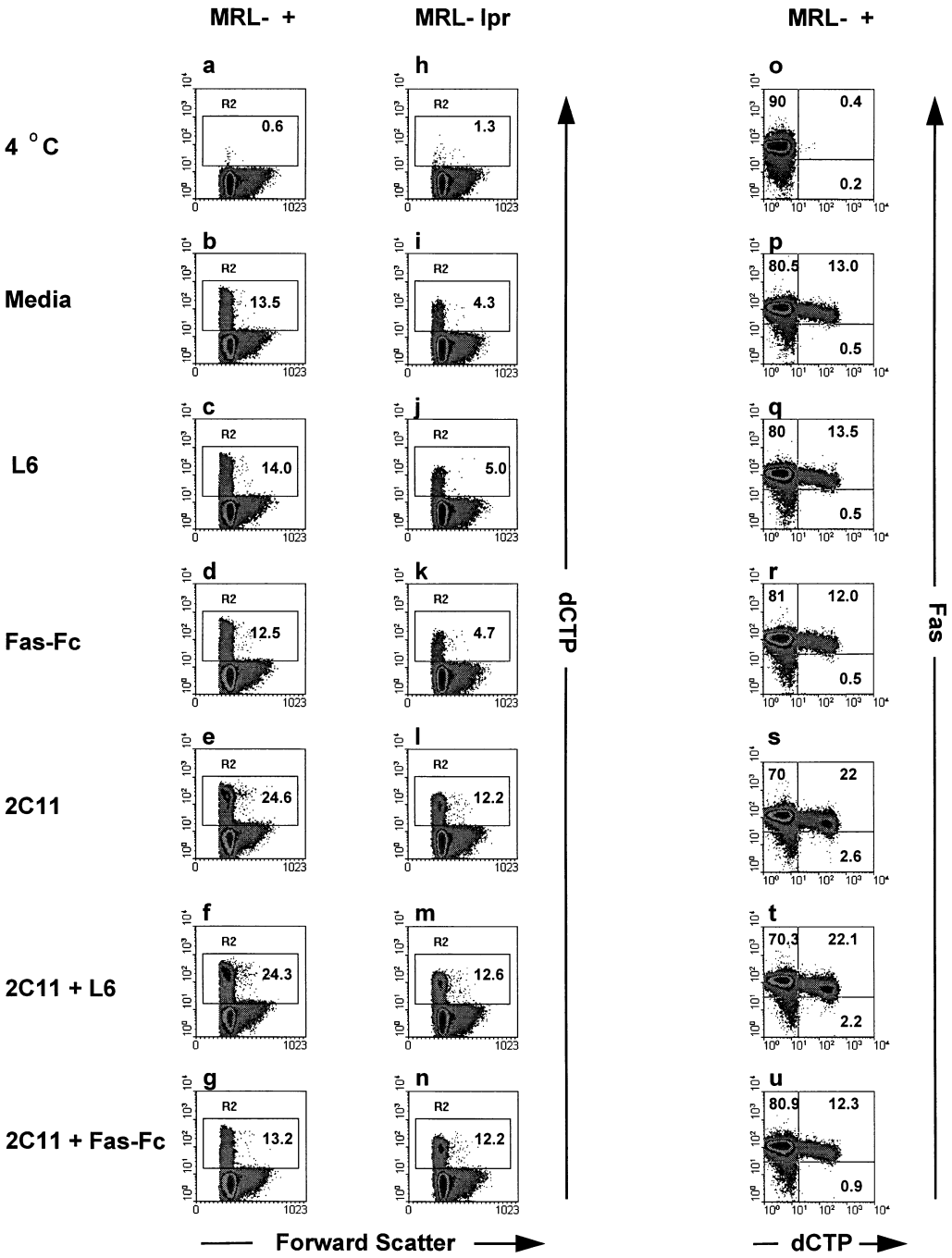

Figure 2. Decreased Susceptibility of MRL-*lpr* Thymocytes to Apoptosis In Vitro

Duplicate samples from two to four mice of MRL-$^+$ (a–g) and MRL-*lpr* (h–n) thymocytes were incubated in RPMI media plus 10% fetal calf serum at 4°C (a and h) or at 37°C (b and i) plus L6, a control human IgG1 MAb (Hellstrom et al., 1986) (c and j); Fas–Fc, a fusion protein composed of the extracellular domain of Fas linked to the Fc region of human IgG1 (Ju et al., 1995) (d and k); anti–TCR CD3ε MAb 145-2C11 (2C11) (Leo et al., 1987) (e and l); 2C11 plus L6 (f and m); or 2C11 plus Fas–Fc (g and n) and analyzed for apoptosis by TdT labeling. MRL-$^+$ (o–u) and MRL-*lpr* (data not shown) thymocytes shown in (a)–(g) were also analyzed for cell surface expression of Fas by flow cytometry. Data are representative of three independent experiments.

negative (DN) thymocytes (Andjelic et al., 1994; Drappa et al., 1993; Nishimura et al., 1995; Ogasawara et al., 1993). Our results show that thymocytes undergoing apoptosis from the MRL-$^+$ strain express high levels of cell surface Fas, whereas few cells lacking Fas expression become apoptotic. This is consistent with previous results showing that anti-Fas MAb can induce apoptosis of thymocytes in vitro (Nishimura et al., 1995; Ogasawara et al., 1995). As expected, the MRL-*lpr* thymocytes did not express detectable levels of Fas (data not shown). These results show that susceptibility to Fas-induced apoptosis correlates with expression of high levels of

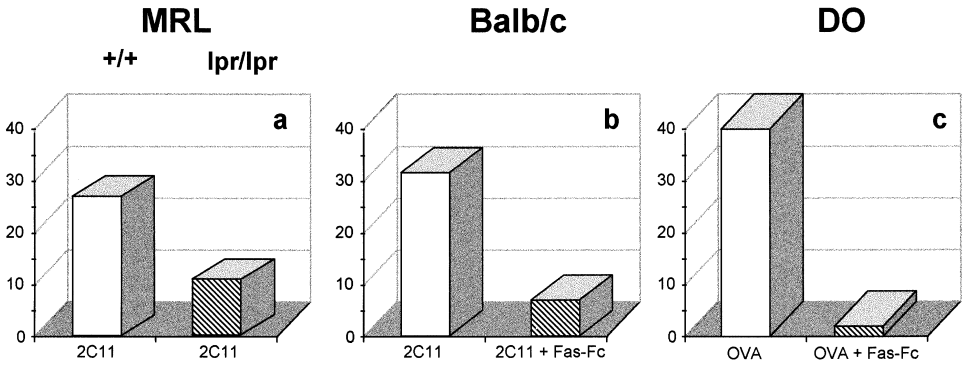

Figure 3. Both Mutant Fas Receptor and Fas–FasL Blockade Inhibit TCR-Induced Apoptosis

(a) We intraperitoneally injected 4- to 6-week-old MRL-$^+$ (open bars) and MRL-*lpr* mice (hatched bars) with 100 μg of 2C11 MAb (2C11) or PBS plus 100 μg of L6 (control). After 10 hr, thymocytes were harvested, labeled with dCTP–Cy5 with TdT, and analyzed by flow cytometry as previously described. The percentage of apoptotic cells was determined in duplicate samples. The percentage of apoptotic cells in PBS control groups (MRL-$^+$, 1.9%; MRL-*lpr*, 2.9%) was subtracted from the 2C11 groups. Data are representative of three independent experiments.

(b) We intraperitoneally injected 4- to 6-week-old BALB/c mice with 100 μg of 2C11 MAb (open bars), 100 μg of 2C11 MAb plus 150 μg of Fas–Fc (hatched bars), or PBS plus 150 μg of L6. After 10 hr, thymocytes were harvested and labeled with dCTP–Cy5 with TdT and analyzed by flow cytometry. The percentage of apoptotic cells in PBS control groups (2.3%) was subtracted from the 2C11 groups. Data are representative of three independent experiments.

(c) We intraperitoneally injected 4- to 6-week-old TCR transgenic DO mice with 1000 μg of OVA324-39 peptide (open bars), 1000 μg of OVA324-39 peptide plus 150 μg of Fas–Fc (hatched bars), or PBS plus 150 μg of L6. After 10 hr, thymocytes were harvested and labeled with dCTP–Cy5 with TdT and analyzed by flow cytometry. The percentage of apoptotic cells in PBS control groups (0.7%) was subtracted from the OVA324-39 peptide groups. Data are representative of two independent experiments.

Fas. However, a subset of the MRL-*lpr* thymocytes did undergo spontaneous apoptosis, and the percentage of cells in this subset was increased after TCR cross-linking, although at consistently lower levels compared with the MRL-$^+$ mice. The simplest interpretation of these results is that both Fas-dependent and Fas-independent mechanisms can regulate apoptosis of thymocytes. However, because low level expression of Fas mRNA has been detected in *lpr* mice (Adachi et al., 1993; Manani et al., 1994), we cannot rigorously exclude the possibility that apoptosis of the MRL-*lpr* thymocytes is due to the expression of functional but undetectable levels of Fas protein.

Thymocytes from *lpr* Mice Have Decreased Sensitivity to TCR-Induced Apoptosis In Vivo

To confirm that the decreased sensitivity of *lpr* thymocytes to TCR-induced apoptosis also occurred in vivo in a normal thymic milieu, we injected MRL-$^+$ and MRL-*lpr* mice with the anti-TCR CD3ε MAb 2C11 (Figure 3a), which has previously been shown to induce apoptosis of thymocytes in vivo (Shi et al., 1991). Consistent with our in vitro results, these data show that the induction of apoptosis of MRL-$^+$ thymocytes (24.2%) was increased compared with MRL-*lpr* thymocytes (9.61%) (Figure 3a). Control mice of both strains injected with phosphate-buffered saline (PBS) had a low incidence of apoptosis that was not significantly different (1.9% and 2.9%, respectively). Thus, thymocytes from *lpr* mice have a decreased sensitivity to TCR-induced apoptosis both in vitro and in vivo.

Blockade of Fas–FasL Interactions Inhibits TCR-Induced Apoptosis in Non-*lpr* Mice

To analyze the role of Fas–FasL interactions in wild-type mice with normal Fas expression, we investigated the effect of blocking Fas–FasL interactions with the Fas–Fc fusion protein in vivo in BALB/c mice (Figure 3b). These results show that MAb 2C11 induced apoptosis in 31.7% of thymocytes. In contrast, mice receiving Fas–Fc plus 2C11 had apoptosis inhibited to 8.7%. Apoptosis in control mice receiving saline was consistently less than 2.3%. Thus, blockade of the Fas–FasL interaction in vivo can inhibit TCR-induced apoptosis of thymocytes in normal mice.

Fas–Fc Inhibits Antigen-Specific Negative Selection In Vivo

To determine the physiological role of Fas-dependent apoptosis of thymocytes, we investigated the effect of blocking Fas–FasL interactions with the Fas–Fc fusion protein in vivo during antigen-specific negative selection using non-*lpr* DO-11.10 (DO) TCR transgenic mice, since previous reports had shown that injection of the OVA protein induces apoptosis of thymocytes in these mice (Murphy et al., 1990). Consistent with the previous reports, the peptide OVA324-39 induced apoptosis of thymocytes from DO mice; importantly, Fas–Fc reduced the percentage of apoptotic thymocytes by >90% (Figure 3c). Thus, blockade of the Fas–FasL interaction can inhibit antigen-specific negative selection in DO TCR transgenic mice.

Fas–Fc Blocks Apoptosis of Fas^{hi}TCR^{int} DP Thymocytes

The deletion of thymocytes during negative selection has been shown by multiple laboratories to occur at the late DP stage in cells expressing intermediate levels of TCR (Swat et al., 1991a). Therefore, we examined the phenotype of the viable and apoptotic thymocytes shown in Figure 3c by four-color flow cytometry for TdT labeling (Figures 4a–4d), expression of TCR (Figures

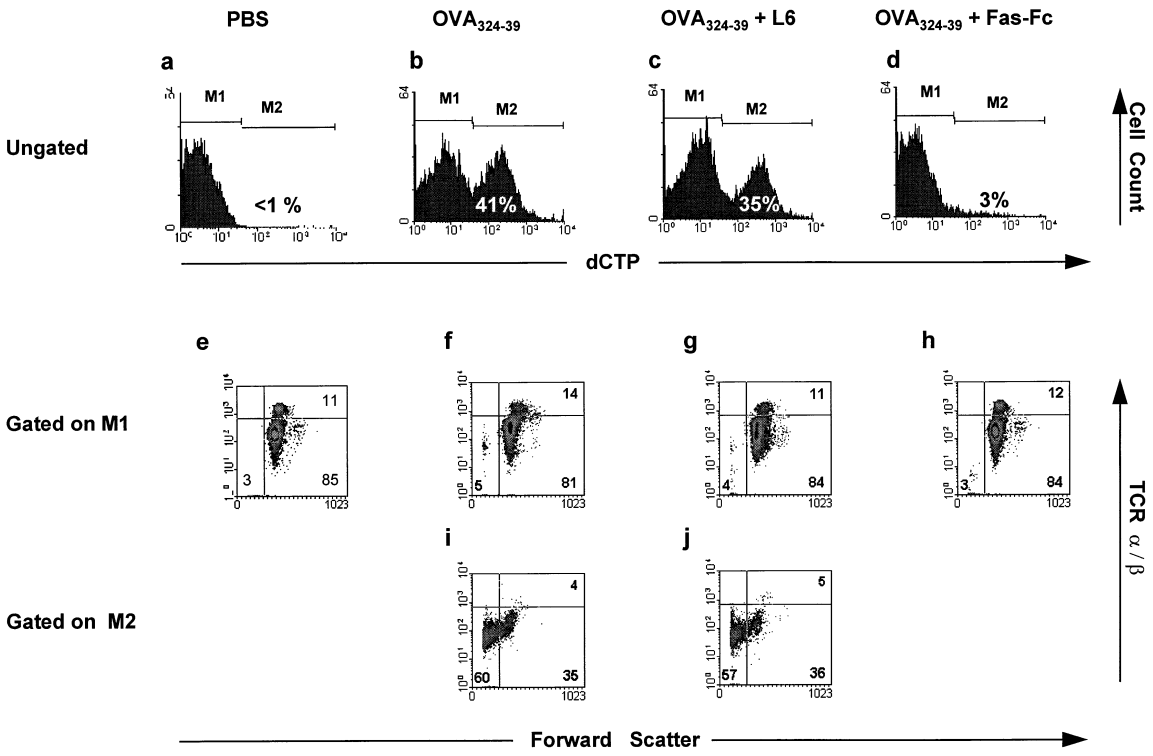

Figure 4. Inhibition of Apoptosis by Fas–Fc during Antigen-Induced Negative Selection In Vivo

Thymocytes from mice given PBS (a), OVA324-39 (b), OVA324-39 plus L6 (c), and OVA324-39 plus Fas–Fc (d) were analyzed for incorporation of fluoresceinated dCTP by TdT labeling (X axis) and cell number (Y axis); gate M1 delineates viable cells, and gate M2 $dCTP^+$ apoptotic cells. Cells from gates M1 and M2 were analyzed for forward scatter (X axis) and TCR expression (Y axis) ([e]–[h] and [i]–[j], respectively). Data are representative of two independent experiments.

4e–4j), and expression of CD4 and CD8 (Figures 5a–5j). Apoptosis detected by TdT labeling of freshly isolated thymocytes from DO mice is consistently <1% (data not shown), a level comparable with thymocytes from mice receiving PBS (Figure 4a). At 12 hr after injection of OVA324-39, 41% of the thymocytes were apoptotic (Figure 4b). In contrast, blockade of Fas–FasL interactions with Fas–Fc resulted in apoptosis of only 3% of the thymocytes following injection of OVA324-39 (Figure 4d). As expected, control L6 did not inhibit apoptosis in OVA324-39-treated mice (Figure 4c).

Next, we analyzed the level of TCR expression of the viable (gate M1) and apoptotic (gate M2) cells shown in Figures 4a–4d. Only 4% and 5% of the apoptotic cells expressed high levels of TCR in mice receiving OVA324-39 or OVA324-39 plus L6, respectively, whereas most of the apoptotic cells (93%–95%) expressed intermediate levels of TCR (Figures 4i and 4j). There were insufficient apoptotic events to analyze in the mice treated with PBS or Fas–Fc (data not shown). In contrast with the apoptotic cells, 11%–14% of the viable cells (gate M1) expressed high levels of TCR (Figures 4e–4h). Together, these results suggest that the thymocytes undergoing apoptosis predominantly express intermediate levels of TCR, which is consistent with previous studies of negative selection.

After 18–24 hr, the percentage of DP thymocytes was markedly decreased (data not shown); however, 12 hr after administration of peptide the percentage of DP (a range of 75%–77%) thymocytes was not significantly different among the four groups (Figures 5a–5d). Analysis of CD4 and CD8 expression showed that the mice treated with OVA324-39 and OVA324-39 plus L6 (Figures 5b and 5c), but not with PBS or OVA324-39 plus Fas–Fc (Figures 5a and 5d), accumulated a population of $CD4^{lo}CD8^{lo}$ cells (gate R2). To confirm that DP thymocytes progress to a $CD4^{lo}CD8^{lo}$ phenotype during apoptosis, we determined the frequency of apoptotic cells within gates R1 and R2. In the mice given PBS or OVA324-39 plus Fas–Fc only 0.4% and 5%, respectively, of the cells within gate R1 were apoptotic (Figures 5e and 5h), whereas 38%–45% were apoptotic in the mice treated with OVA324-39 or OVA324-39 plus L6 (Figures 5f and 5g). Strikingly, 91% and 93% of the thymocytes expressing decreased levels of CD4 and CD8 (gate R2) from mice receiving OVA324-39 (Figure 5i) or OVA324-39 plus L6 (Figure 5j), respectively, were apoptotic. Again, there were insufficient apoptotic events in the Fas–Fc-treated group to analyze CD4 and CD8 expression (data not shown). Analysis of the forward scatter of cells within gate R2 shows that the $CD4^{lo}CD8^{lo}$ cells are smaller than the population within gate R1 (Figures 5i and 5j). Together, these results suggest that the apoptotic cells progress from larger cells within gate R1 to smaller cells within gate R2. Previous reports showed that thymocytes undergoing apoptosis express lower levels of CD4 and CD8 (Kishimoto et al., 1995; Swat et al., 1991b). Our analysis confirms reduced levels of CD4 and CD8, but

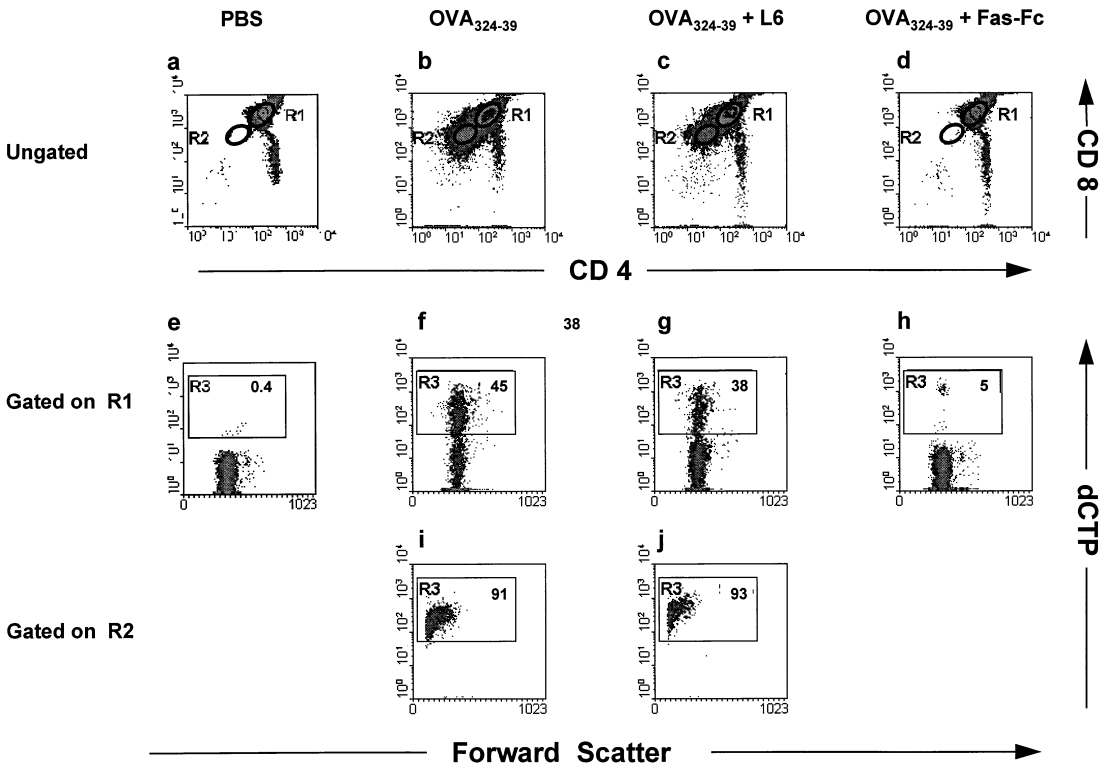

Figure 5. Fas–Fc Inhibits Apoptosis of the TCR^{int}CD4^{lo}CD8^{lo} DP Thymocytes In Vivo during Antigen-Induced Negative Selection

Using list-mode data, the same cells shown in Figure 4 were also analyzed for expression of CD4 (X axis) and CD8 (Y axis) (a–d). CD4^{hi}CD8^{hi} (gate R1) and CD4^{lo}CD8^{lo} (gate R2) thymocytes were analyzed by forward scatter (X axis) and dCTP incorporation (Y axis) ([e]–[h] and [i]–[j], respectively); gate R3 includes cells labeled with dCTP–Cy5 (percentage of apoptotic cells is shown). Data are representative of two independent experiments. Four DO TCR transgenic mice were injected intraperitoneally with PBS (a and e), 1 mg of OVA324-39 (b, f, and i), 1 mg of OVA324-39 plus 150 μg of L6 (e, g, and j), or 1 mg of OVA324-39 plus 150 μg of Fas–Fc (d and h). After 12 hr, thymocytes were harvested and processed as previously described, except that the cells were stained with anti-TCRαβ–phycoerythrin, anti-CD4–FITC, and anti-CD8–biotin plus red 613 in addition to TdT labeling with dCTP–Cy5.

indicates that the decreased fluorescence correlates with decreased size. In conjunction with the analysis of TCR expression, these results indicate that apoptotic thymocytes are predominantly CD4^{lo}CD8^{lo} cells that express intermediate levels of TCR. Previous observations had shown that DP thymocytes are particularly susceptible to induction of apoptosis by anti-Fas MAb or FasL-expressing cells (Arase et al., 1994; Nishimura et al., 1995). Our results show that Fas–Fc inhibits apoptosis and the appearance of the CD4^{lo}CD8^{lo} DP thymocytes in vivo during antigen-induced negative selection.

Morphology of Apoptotic Thymocytes

To analyze the morphology of the apoptotic thymocytes in conjunction with cell surface expression of TCR, cytospin preparations were made from thymocytes from DO mice given OVA324-39 plus L6 or OVA324-39 plus Fas–Fc (Figure 6). Examination with Nomarski optics showed an increased proportion of small thymocytes from mice administered OVA324-39 (Figure 6b) compared with OVA324-39 plus Fas–Fc (Figure 6a). Analysis of the same slides by immunofluorescence to detect apoptotic cells by dCTP incorporated with TdT labeling showed increased apoptotic cells in the mice receiving peptide alone (Figure 6d) compared with the mice receiving Fas–Fc plus peptide (Figure 6c). Most of the apoptotic cells correspond to the small thymocytes observed with the Nomarski optics. As expected, dual color immunofluorescence to detect TCR expression in addition to dCTP incorporation showed that viable cells with and without detectable TCR expression were present (Figures 6e and 6f). Importantly, all of the observed apoptotic cells expressed detectable levels of TCR (Figure 6f). The expression of TCR on the apoptotic cells is consistent with our previous results (Figures 2–4) showing that TCR signals promote Fas-dependent apoptosis. In addition, these data are consistent with previous results showing that during apoptosis cells decrease in size owing to condensation and blebbing.

Fas–Fc Inhibits Apoptosis of Predominantly Cortical Thymocytes

Localization of apoptosis within the thymus was determined by immunohistochemistry with TdT-mediated dUTP-digoxigenin nick-end labeling (TUNEL) (Figure 7). Thymus sections from DO mice that received PBS showed low levels of basal apoptosis (Figure 7c), whereas mice that received OVA324-39 plus L6 had increased apoptosis (Figure 7b). In contrast, the mice that received OVA324-39 plus Fas–Fc had levels of apoptosis that were similar to the PBS control (Figure

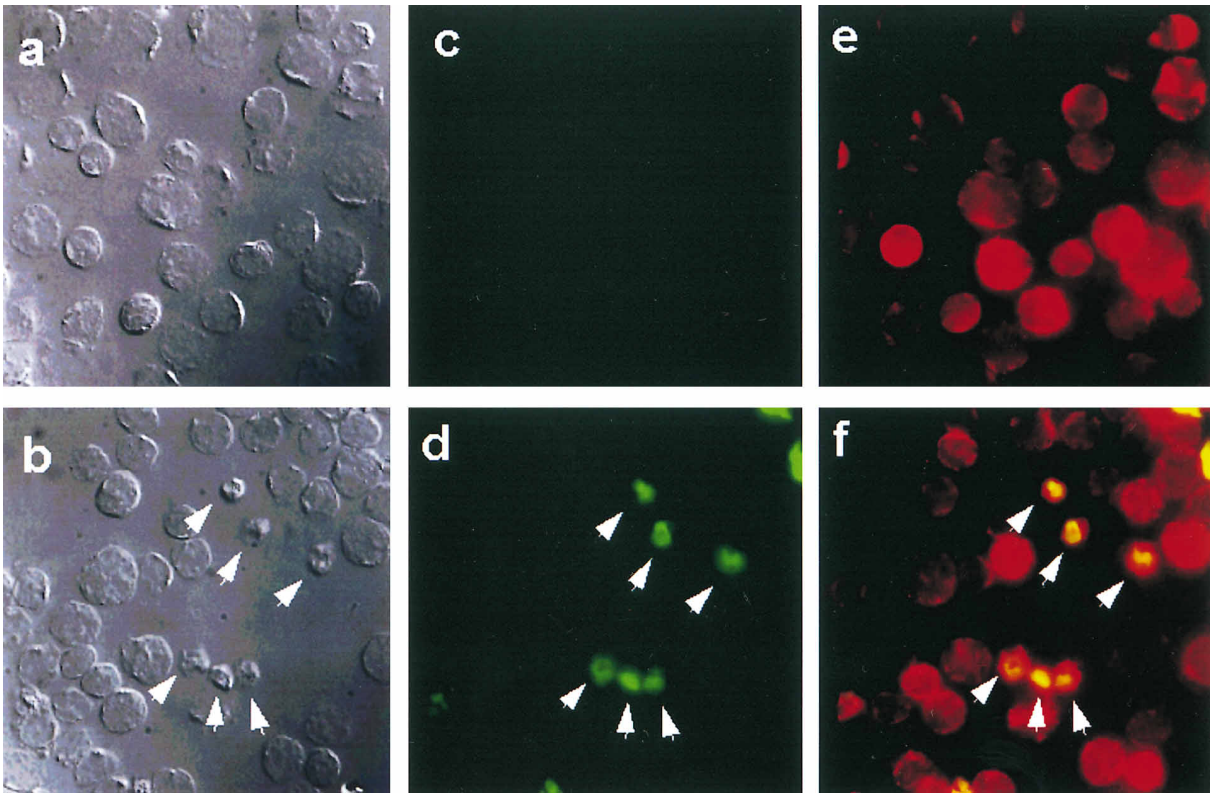

Figure 6. Differential Interference Contrast and Immunofluoresence Microscopy of Apoptotic Cells

Thymocytes from DO mice treated with OVA324-39 plus Fas–Fc (a, c, and e) or OVA324-39 plus L6 (b, d, and f) were stained with phycoerythrin-labeled anti-TCR$\alpha\beta$ MAb H57-59 (Pharmingen) and then labeled with dCTP–biotin plus streptavidin–FITC by TdT as described in Figure 1. Arrowheads indicate small cells (b) that incorporated dCTP (d) and labeled with anti-TCR MAb (f). Slides were prepared with a Cytospin centrifuge. The samples were then visualized with a differential interference contrast microscope (Nikon Diaphot 300) using the Nomarski-DCI (a and b) and Diaphot EPI-fluorescence attachments for fluorescein (green) (c and d) and phycoerythrin (red) and fluorescein (green) fluorescence (e and f). The images were processed and analyzed using the Oncor Image System. Data are representative of two independent experiments.

7a). This effect was evident as early as 4 hr and at least up to 12 hr after treatment (data not shown).

Consistent with previous reports, the apoptotic cells often appeared in clusters, and the size of the clusters increased with OVA324-39 treatment, probably owing to engulfment by phagocytic macrophages (Kishimoto

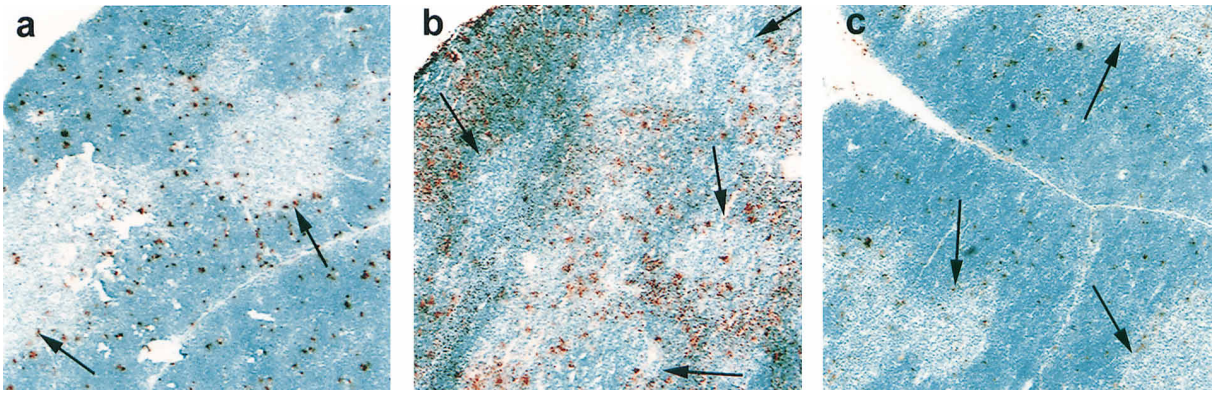

Figure 7. Fas–Fc Inhibits Apoptosis of Predominantly Cortical Thymocytes

Thymi were harvested from DO mice 4 hr after receiving PBS (c), OVA324-39 plus L6 (b), or OVA324-39 plus Fas–Fc (a). Duplicate 6 μm cryosections of mouse thymus were stained by the TUNEL method according to the Apotag in situ apoptosis detection protocol or without TdT enzyme to control for endogenous peroxidase activity (Oncor) (Gavrieli et al., 1992). Slides were developed with diamino-benzidine as the substrate. Sections were counterstained with methyl green. Apoptotic cells are stained brown. Images were analyzed at 4$\times$ magnification. Septae are apparent in the sections. Arrows indicate medullary regions.

et al., 1995; Liblau et al., 1996). Apoptosis in all three groups was localized predominantly in the cortex, with infrequent apoptotic cells visualized in the medullary regions. These results show that Fas–Fc inhibits antigen-induced apoptosis of thymocytes that are predominantly localized to the cortical regions.

Discussion

Our results show that thymocytes from *lpr* mice have decreased susceptibility to TCR-induced apoptosis. To confirm the physiological significance of this observation, we also demonstrated that blocking Fas–FasL signals inhibits antigen-induced apoptosis of thymocytes during negative selection in vivo. Differential interference contrast and immunofluorescence microscopy showed that Fas–FasL blockade inhibited progression of thymocytes to small TCR^+ apoptotic cells. Using four-color flow cytometry, we showed that Fas–FasL blockade inhibited apoptosis of $Fas^{hi}TCR^{int}CD4^+CD8^+$ DP thymocytes. In addition, based on immunohistochemistry with the TUNEL assay, Fas–FasL blockade inhibited apoptosis of thymocytes that were predominantly located in the cortical regions. Negative selection commonly occurs at the $Fas^{hi}TCR^{int}CD4^+CD8^+$ DP stage of thymocyte development in the cortex or at the cortical-medullary junction. Thus, all of our results are in agreement with a model in which Fas–FasL interactions can modulate apoptosis of thymocytes during negative selection.

Consistent with our results, several studies have shown decreased deletion of thymocytes in *lpr* mice. For example, Mountz and coworkers (1994) noted decreased deletion of CD8 SP thymocytes in male C57Bl/6-*lpr* compared with C57Bl/6^+ H-Y TCR transgenic mice (44% and 10%, respectively). Also, decreased deletion of *lpr*-derived thymocytes was observed in chimeric mice (Matsumoto et al., 1991), and increased emigration of thymocytes was detected in *lpr* mice following tolerance induction (Zhou et al., 1993). Additionally, in in vitro studies Fas signals have been shown to induce apoptosis of thymocytes (Ogasawara et al., 1995; Yonehara et al., 1994; Fisher et al., 1996). However, a number of previous studies did not detect Fas-dependent deletion of thymocytes (Adachi et al., 1996; Giese and Davidson, 1992; Herron et al., 1993; Kotzin et al., 1988; Mountz et al., 1990; Musette et al., 1994; Sidman et al., 1992; Singer and Abbas, 1994; Singer et al., 1994; Singer et al., 1989; Sytwu et al., 1996). The prior studies analyzing TCR Vβ deletion in *lpr* mice investigated thymocyte deletion by endogenous superantigens, suggesting the possibility that Fas modulates apoptosis induced by peptide antigens, but not superantigens. However, this is unlikely based on results showing that the cytochrome and hemagglutinin peptides induce thymocyte deletion in TCR transgenic *lpr* models (Singer and Abbas, 1994; Sytwu et al., 1996). Another possibility is that Fas modulates apoptosis of thymocytes only in specific mouse strains. We analyzed two different genetic backgrounds and obtained similar results. Therefore, it is unlikely that our results differ with those of others because of the unique characteristics of a specific strain. In addition, our results in both the MRL-$^+$ and BALB/c strains using the anti–TCR CD3ϵ MAb (2C11) that recognizes all TCRs expressing the CD3 complex strongly support our argument that modulation of thymocyte apoptosis by Fas–FasL interactions is not limited to a small subset of TCRs or to a narrow range of TCR/peptide–MHC avidities. Another explanation for our detection of apoptotic cells may be the increased sensitivity of detecting TdT-labeled apoptotic cells by flow cytometry compared with other methods including ladder gels and fluorescent staining of subdiploid DNA content. Our results show that TdT labeling can detect apoptotic cells with normal size parameters before they become smaller than viable cells. Thus, our use of the TdT method combined with flow cytometry may partially account for our detection of Fas modulation of thymocyte apoptosis; however, it is unlikely this is sufficient to account completely for the difference between our results and previous studies.

A major difference between our experiments and previous studies is the kinetics of the analyses. Our experiments focused on early timepoints, including 1.5–2 hr in in vitro assays and 4–12 hr in in vivo experiments, whereas none of the prior studies reporting that Fas had no effect on thymocyte deletion examined timepoints earlier than 24 hr. For example, some studies showed the predicted deletion of specific TCR Vβ chains by endogenous superantigens in adult *lpr* mice (Adachi et al., 1996; Giese and Davidson, 1992; Herron et al., 1993; Kotzin et al., 1988; Mountz et al., 1990; Musette et al., 1994; Singer et al., 1989) or mice made deficient in Fas by homologous recombination (Adachi et al., 1996). Peptide antigens have also been shown to delete thymocytes in TCR transgenic *lpr* mice. For example, Singer and Abbas (1994) analyzed thymocyte deletion in 2B4 TCR transgenic *lpr* mice at day 7 after three injections of peptide antigen. Their results showed comparable deletion in *lpr* and normal strains analyzed at day 7. Also, Sytwu et al. (1996) analyzed the HNT TCR transgenic *lpr* strain and found comparable deletion by peptide antigen in *lpr* and normal strains between 1 and 7 days after peptide injection. However, none of these experiments analyzed timepoints earlier than 24 hr. At the earlier timepoints analyzed in our studies, we detected quantitative differences between MRL-*lpr* and MRL-$^+$ strains. Also, in experiments investigating *lpr* mice, Fas-independent mechanisms could at least partially compensate for the deficiency of Fas and promote thymocyte deletion. Together, these observations support a model in which Fas signals modulate TCR-induced apoptosis at early timepoints. It is possible that Fas-independent signals modulate apoptosis at later timepoints.

In addition to our results demonstrating that Fas can modulate negative selection of thymocytes, two other members of the TNFR family, CD40 and CD30, have recently been shown to modulate apoptosis of thymocytes. In models of negative selection, deletion was blocked by the loss of gp39 function when the antigen or superantigen was endogenously expressed, but not when the antigen was exogenously administered (Foy et al., 1995). Interestingly, our results are reciprocal. Blockade of Fas–FasL inhibits deletion in response to exogenous antigens, whereas several groups have

shown normal deletion to endogenous superantigens in *lpr* mice (Adachi et al., 1996; Giese and Davidson, 1992; Herron et al., 1993; Kotzin et al., 1988; Mountz et al., 1990; Musette et al., 1994; Sidman et al., 1992; Singer and Abbas, 1994; Singer et al., 1989). In CD30-deficient mice, negative selection was shown to be impaired to endogenous antigens; however, the effect of exogenous antigens was not evaluated (Amakawa et al., 1996). Together, these observations suggest the hypothesis that different members of the TNFR family may modulate apoptosis of thymocytes in different contexts, such as different stages of development or in response to different signals. As indicated by our results, only Fas has been shown to block apoptosis of thymocytes in vivo in response to exogenous antigens.

A recent report showing that Fas and the p55 TNFR may cross-talk by cytoplasmic protein–protein interactions involving MORT1 (FADD) and TRADD (Varfolomeev et al., 1996) suggests a model in which multiple signals transduced by members of the TNFR/TNF families (including Fas/FasL, CD30/CD30L, CD40/gp39, and TNFR) could be integrated by cross-talk into one signal that modulates apoptosis. In this model, signals by members of the TNFR family could have redundant, or at least partially overlapping, functions. Relevant to this notion, *lpr* mice that have a congenital Fas deficiency may develop compensatory mechanisms to regulate thymocyte deletion that are not functional in our experiments analyzing non-*lpr* mice (for example, the CD30–CD30L, CD40–gp39, or TNFR–TNF receptor–ligand pairs could be overexpressed in *lpr* mice). Consistent with this hypothesis, splenic and thymic cells from *lpr* mice express increased quantities of TNFα compared with non-*lpr* controls (Tsai et al., 1995). Alternatively, the signals by members of the TNFR family could function independently. It will be important to determine whether the Fas-dependent and Fas-independent signals are integrated into a single response and coordinately regulate apoptosis or whether they mediate apoptosis in different subsets of thymocytes during different stages of maturation and selection.

Previous reports showed that Fas was important in the regulation of TCR-induced apoptosis of mature peripheral T cells (Brunner et al., 1995; Dhein et al., 1995; Ju et al., 1995; Singer and Abbas, 1994; Sytwu et al., 1996). More recently, TNF has also been shown to induce apoptosis of both CD8 and CD4 T cells (Sytwu et al., 1996; Zheng et al., 1995). Thus, at least two members of the TNFR family can regulate apoptosis of mature T cells. Previous reports have also implicated members of the TNFR/TNF families, including CD30/CD30L and possibly CD40/gp39, in the regulation of apoptosis of thymocytes. In this report our results demonstrate that Fas-dependent signals can modulate apoptosis of thymocytes during early phases of negative selection. Thus, it is likely that multiple receptors can regulate apoptosis of thymocytes. In future studies, it will be important to understand the roles of Fas-dependent and Fas-independent apoptosis of thymocytes.

Experimental Procedures

Mice

All mice used were 4–6 weeks old and were housed in virus antibody–free facilities. Animals were maintained in accordance with the guidelines of the Committee on Animals of Harvard Medical School, and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. BALB/c, MRL-$^{+/+}$ (MRL-$^{+}$), and MRL-*lpr/lpr* (MRL-*lpr*) mice were purchased from Jackson Laboratories (Bar Harbor, ME). DO-11.10 (DO) TCR transgenic mice, specific for the OVA324-39 peptide in association with I-A^{d}, were originally provided by Dr. D. Loh (Washington University, St. Louis, MO) and bred in our animal facility.

Peptides

The peptide OVA324-39 (SQAVHAAHAEINEAGR) was synthesized by standard Fmoc chemistry using an Applied Biosystems biopolymer synthesizer at the Brigham and Women's Hospital Biopolymer Laboratory. All peptides were purified by high pressure liquid chromatography. Composition was confirmed by amino acid analysis using a 6300 Amino Acid Analyzer (Beckman) and plasma-desorption time-of-flight mass spectroscopy.

Activation of Thymocytes In Vitro

Thymocytes were prepared as previously described. In brief, duplicate samples of MRL-$^{+}$ and MRL-*lpr* thymocytes from two to four mice were incubated at 2×10^5 cells per well in media plus 10% fetal calf serum at 4°C (negative control) or 37°C plus 20 μg/ml L6, an isotype-matched control human IgG1 MAb (Hellstrom et al., 1986), 20 μg/ml Fas–Fc, a fusion protein composed of the extracellular domain of Fas linked to the Fc region of human IgG1 (Ju et al., 1995), 20 μg/ml immobilized anti-TCR CD3ϵ MAb 145-2C11 (2C11) (Leo et al., 1987), 20 μg/ml 2C11 plus 20 μg/ml L6, or 20 μg/ml 2C11 plus 20 μg/ml Fas–Fc for 1.5 hr.

Antigen-Specific Deletion of Thymocytes In Vivo

BALB/c, MRL-*lpr*, or DO TCR transgenic mice were injected intraperitoneally with 100 μg of 2C11 MAb or 1 mg of OVA324-39 peptide in PBS alone or with 150 μg of Fas–Fc or 150 μg of L6, and thymi were harvested at the indicated times.

Flow Cytometry

Freshly isolated thymocytes were stained with fluorescent labeled MAb and analyzed by flow cytometry as previously described (Perkins et al., 1996). In brief, 1×10^6 cells were incubated with saturating concentrations of fluorescent antibodies in 50 μl for 30 min at 4°C in PBS, 1% mouse serum, 5% bovine calf serum, and 0.1% NaN_3. The cells were then fixed in 1% paraformaldehyde in PBS (pH 7.4) for 15 min and treated with 100% ethanol for 3 hr at −20°C. Cells were washed twice and then incubated at 37°C for 15 min with 5 U of TdT (Promega, Madison, WI), and 6.25 pmol per well dCTP–Cy5 (Amersham, Arlington Heights, IL) in 1× TdT buffer (Promega). Cells were washed and 1×10^4 to 5×10^4 events were analyzed with Lysis software on a Becton Dickinson FACS Vantage using 488 (fluorescein isothiocyanate [FITC], phycoerythrin [PE], and red 613) and 633 (Cy5) nm excitation wavelengths. Fluorescence was detected at 525 (FITC), 590 (PE), 613 (red 613), and 670 (Cy5) nm. Graphics were prepared using the WinMDI software (Joseph Trotter, Scripps Institute, La Jolla, CA).

MAbs

The MAbs specific for CD4 (GK1.5; Dialynas et al., 1983) and CD8 (53.6; Ledbetter and Herzenberg, 1979) were purified from hybridoma supernatant by passing over a protein A column and eluted with 125 mM sodium acetate. Fractions were immediately neutralized to pH 8 by titrating 1 M Na_2CO_3. Purified MAb was conjugated to FITC and long-arm biotin, respectively (Sigma, St. Louis, MO). In brief, 1 mg of purified MAb was alkalinized by the addition of 1 M Na_2CO_3 (pH 9.4; 1:10 [v/v]) to make a 100 mM Na_2CO_3 buffered solution. We added 100 μg of conjugate dissolved in anhydrous DMSO per milligram of MAb for 30 min at room temperature. Free dye was separated from labeled MAb by elution through a gel filtration column (G50 Sephadex) using PBS plus 0.02% NaN_3. Anti-Fas and anti-TCRβ were obtained from Pharmingen (San Diego, CA).

Differential Interference Contrast and Immunofluorescence Microscopy

Freshly isolated thymocytes were stained with fluorescent MAb and then labeled with dCTP–biotin plus streptavidin–FITC by TdT as described. Slides previously coated with Biobond (Goldmark Biologicals, Phillipsburg, NJ) were prepared by spinning 5×10^5 cells at 500 rpm for 5 min using a Cytospin centrifuge. Coverslips were mounted on air-dried slides with Vectashield (Vector Laboratories Inc., Burlingame, CA). The samples were then visualized with a differential interference contrast microscope (Nikon Diaphot 300) using the Nomarski-DCI and Diaphot EPI-fluorescence attachments for fluorescein (green) and PE (red) fluorescence. The images were processed and analyzed using the Oncor Image System (Gaithersburg, MD).

Immunohistochemistry

Duplicate 6 μm cryosections of mouse thymus were stained by the TUNEL method according to the Apotag in situ apoptosis detection protocol (Oncor). Control sections were stained without the TdT enzyme to control for endogenous peroxidase activity. Slides were developed with diamino-benzidine as the substrate. Sections were counterstained with methyl green. The microscopy images were processed and analyzed using the Oncor Image System.

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